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- 1) Crompton et al. Journal of General Virology. 1994; 75 (pt 1): 133-139.
- 2) Yang et al. Human Gene Therapy. 1998; 9/13: 1929-1937.
- 3) Douglas et al. Nature Biotechnology. Nov. 1996; 14 (11): 1574-1578.
- 4) Hallenbeck et al. (Advances in Experimental Medicine and Biology. 2000; 465: 37-46.

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Expression of a foreign epitope on the surface of the adenovirus hexon

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To present short protein sequences to the host immune system a foreign epitope has been expressed on the surface of the adenovirus virion as part of the hexon. As the trimeric hexon constitutes 240 out of the 252 capsomers of the virus, the foreign epitope is repeated 720 times on the virion surface. An eight amino acid sequence from the major antigenic site in the VP1 capsid protein of poliovirus type 3 was engineered into two regions of the adenovirus type 2 hexon. The two loop regions chosen to accommodate the foreign sequences are exposed on the surface of the virion, show sequence variation between serotypes and are the sites of

interaction with neutralizing antibodies. Virus with substitutions in loop I had wild-type growth characteristics, whereas virus with substitutions in loop II grew poorly. Adenoviruses with poliovirus sequences in loop I were recognized and efficiently neutralized by antisera specific for the poliovirus sequence; an antiserum raised against the adenovirus with the poliovirus insert specifically recognized the VP1 capsid protein of poliovirus type 3. It is therefore feasible to alter the surface properties of the adenovirus virion and in doing so to manipulate the immune response to this virus.

Introduction

Human adenoviruses, of which 47 distinct serotypes have been identified (Hierholzer *et al.*, 1988), replicate in the mucosal surfaces of respiratory, ocular and gastrointestinal tissues giving rise to a variety of acute infections. For over 30 years protection against infection by these viruses has been achieved by oral immunization which results in the generation of strong mucosal immunity. Live adenovirus types 4 and 7 (Ad4 and –7) in enteric coated capsules have been administered to military personnel to prevent acute respiratory disease with no indication of adverse reactions. Oral immunization with serotypes 1, 2 and 5 has also proved effective in clinical trials (reviewed in Ginsberg, 1984).

Adenoviruses are non-enveloped and contain a linear double-stranded DNA genome of approximately 36 kb that is packaged within an icosahedral capsid. Hexon is the major structural component of the virus capsid, forming the 20 facets of the icosahedron; the vertices are composed of a complex of penton base and fibre (Ginsberg, 1984). Type-specific antigenic determinants of hexon have been demonstrated on the surface of the virion (Willcox & Mautner, 1976*a, b*) but the antigenic structure is complex with monoclonal antibodies (MAbs) defining at least 19 different epitopes on the surface of hexon (Russell *et al.*, 1981; Adam *et al.*, 1986). Sequence comparison of human adenovirus hexons and alignment with the three-dimensional structure of the adenovirus

type 2 (Ad2) hexon (Roberts *et al.*, 1986) indicates that although the base of the hexon is very highly conserved the surface loops are highly variable (Kinloch *et al.*, 1984; Toogood & Hay, 1988; Toogood *et al.*, 1989). The prediction that these variable regions could represent type-specific neutralizing determinants was confirmed by the demonstration that antibodies raised against synthetic peptides derived from the loop regions could neutralize virus in a type-specific fashion (Toogood *et al.*, 1992).

The variability in the length and sequence of these exposed loops suggested that they could be manipulated to express foreign antigenic determinants. This approach has been successfully used to generate a range of polioviruses with altered antigenic properties. Although the initial antigenic hybrids contained sequences from other poliovirus serotypes a wide range of epitopes from other pathogens have now been incorporated into poliovirus antigenic chimeras (Burke *et al.*, 1988, 1989; Colbere-Garapin *et al.*, 1988; Dedieu *et al.*, 1992; Evans *et al.*, 1989; Jenkins *et al.*, 1990; Kitson *et al.*, 1991; Lémon *et al.*, 1992; Martin *et al.*, 1988; Minor *et al.*, 1990, 1991; Murdin & Wimmer, 1989; Murdin *et al.*, 1991*a, b*, 1992; Murray *et al.*, 1988*a, b*; Reimann *et al.*, 1991). Although the relatively small size and ease of manipulation of polioviruses make them useful models for this type of work, the increased genetic stability of DNA viruses and the expanding use of adenoviruses as gene delivery vehicles make the construction of adeno-

viruses with altered surface properties an attractive proposition.

The objective of this work was to construct adenoviruses expressing foreign antigenic determinants on the surface of the virion. A type-specific antigenic determinant from poliovirus type 3 was engineered into the surface loops of the Ad2 major surface protein, hexon. One of the chimeric adenoviruses grew well in tissue culture and was neutralized by antisera specific for the inserted poliovirus epitope. An antiserum raised against the adenovirus with the poliovirus insert specifically recognized the VP1 capsid protein of poliovirus type 3.

Methods

Cells and viruses. HeLa cells were grown in suspension in Glasgow-modified spinner Eagles' medium (GS-MEM, Gibco) with 7% newborn calf serum. Adenovirus virions were prepared from infected HeLa cells by fluorocarbon extraction of freeze-fractured cells, followed by density gradient centrifugation (Hay *et al.*, 1984). Virus was stored at -20°C in 50% glycerol. The titre of the virus stock was in the region of 10^{11} to 10^{12} p.f.u. per ml. Adenovirus type 5 (Ad5) ts 2 was obtained from Dr V. Mautner, MRC Virology Unit, Glasgow, U.K. and was propagated at 32°C . Virus titres at the permissive and non-permissive temperatures were determined by plaque assay on A549 cells as described previously (Williams, 1970). 293 cells (Graham *et al.*, 1977) were grown as monolayers in G-MEM containing 10% fetal calf serum (FCS). Vaccine strains of poliovirus types 1, 2 and 3 were obtained from Dr M. Ferguson, National Institute for Biological Standards and Control, Potters Bar, U.K. and were propagated and titrated in HeLa cells. Monolayer cultures of A549 cells, a human cell line derived from lung carcinoma, were grown in G-MEM supplemented with 10% FCS.

Construction of recombinant viruses. The Ad2 *Hind*III A fragment cloned in pAT153 (Mautner & Boursnell, 1983) was obtained from Dr V. Mautner. Plasmid DNA was cleaved with *Sma*I which has two recognition sites in the plasmid at positions 51.8 and 56.0 in the adenovirus sequences (Fig. 1) and the two fragments were isolated from an agarose gel. The small 51.8 to 56.0 fragment was cloned into *Sma*I-cleaved M13mp8 and single-stranded DNA was prepared as a substrate for mutagenesis. Two long oligonucleotides were synthesized containing the sequence to be substituted into the hexon gene at either loop I or loop II (Fig. 1) flanked by the appropriate complementary sequence and annealed to the M13 single-stranded DNA described above. The DNA was extended by *Escherichia coli* DNA polymerase I (large fragment) and sealed in the presence of T4 DNA ligase. Double-stranded circular DNA was excised from an agarose gel and used to transform *E. coli* JM101. A short oligonucleotide, labelled at the 5' end with ^{32}P , homologous only to the poliovirus sequences was used to identify recombinants in a plaque screen. Single-stranded DNA from individual plaques was sequenced by the dideoxynucleotide procedure using primers complementary to sequences present in the flanking Ad2 hexon region. To insert these sequences back into the Ad2 hexon gene, double-stranded M13 DNA containing the desired insertion was cleaved with *Sma*I, ligated to the *Sma*I-cleaved large fragment from the *Hind*III A clone and plasmids containing the poliovirus inserts were identified by colony hybridization with the ^{32}P -labelled oligonucleotides described above. Integrity of inserts was confirmed by sequencing as described above. The modified hexon gene was reconstructed into the adenovirus genome by homologous recombination *in vivo*. To improve the efficiency with which recombinants were recovered use was made of the Ad5 temperature-sensitive mutant ts 2 which has a lesion in the

hexon gene. Recombination with the Ad2 hexon gene containing the poliovirus sequences should rescue infectivity of the ts mutant at the non-permissive temperature. DNA from Ad5 ts 2 virions and the modified *Hind*III A plasmid were cotransfected into 293 cells at the permissive temperature of 32°C as described (Hay *et al.*, 1984) and virus was harvested after 5 days. The virus yield was titrated at 32°C and 38.5°C . Plaques arising at 38.5°C were inoculated into 24-well plates and viral DNA was tested for the presence of poliovirus sequences by PCR analysis. Isolates Ad5 PLI and Ad5 PLII contain the poliovirus sequences within the regions encoding hexon loops I or II.

Antisera. Two female Dutch rabbits were immunized subcutaneously with 50 μg of purified, heat-inactivated Ad5 PLI virions in Freund's complete adjuvant. Rabbits were boosted after 2 weeks with 50 μg virions in Freund's incomplete adjuvant, a test bleed being taken 2 weeks later and tested by ELISA for specific antibody. After a second boost serum was collected from each rabbit. Anti-poliovirus peptide antisera R77, R78, R126, R166, R222 and R235 raised in rabbits were obtained in freeze-dried form from Dr M. Ferguson and once rehydrated were used at the indicated dilutions. MAb R2/76 directed against the Ad2 hexon was obtained as an ascitic fluid from W. C. Russell (St Andrews).

Western blots. Proteins (20 μg) were separated in a 10% SDS-polyacrylamide gel and transferred to nitrocellulose using a semi-dry blotter. Non-specific binding sites on the membrane were blocked with 10% non-fat milk, the membrane was exposed to the primary rabbit antibody (1:3000 dilution), washed and exposed to a 1:3000 dilution of affinity-purified donkey anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Amersham). The blot was developed using enhanced chemiluminescence (Amersham).

ELISA. The reactivity of specific anti-peptide antibodies with Ad2 or Ad5 PLI was measured by ELISA using an HRP detection system. Microtitre plates (96-well) were coated overnight at 4°C with 0.5 μg per well of purified adenovirus virions in PBS. After washing with PBS, non-specific binding sites were blocked by incubation with PBS containing 10% low-fat milk (Marvel) and 0.1% sodium azide for 3 h at 37°C . Doubling dilutions of each antiserum were allowed to react with the immobilized virus for 1 h at room temperature. After washing, plates were incubated with HRP-conjugated goat anti-rabbit IgG or a similarly conjugated anti-mouse IgG for 1 h at 37°C , and the colour was subsequently developed with ortho-phenylenediamine and hydrogen peroxide for 30 min in the dark. Microtitre plates were read in a Titertek Multiskan. Specificity was assessed by substituting the immune serum with the corresponding pre-immune serum.

Neutralization test. Appropriate dilutions of virus were determined that would cause a visible c.p.e. in a microtitre well of A549 cells following 3 days (poliovirus) or 5 days (adenovirus) infection. Thus for neutralization tests 50 μl of a dilution of virus stock was mixed with doubling dilutions (starting at 1:10) of each antiserum in serum-free medium. Viruses and sera were incubated for 1 h at 37°C and absorbed onto A549 cells in a microtitre plate for a further 1 h at 37°C . GS-MEM containing 2% FCS was added and incubation continued for either 3 or 5 days at 37°C . Monolayers were fixed with formaldehyde and stained with crystal violet.

Results

Introduction of a poliovirus epitope into the adenovirus hexon

The objective of this work was to present foreign epitopes to the host immune system by their expression as part of the adenovirus major capsid protein, hexon.

(a) 50-1
HindI

(b) LI
N T T
AAT ACT ACI

PLI
N L D
AAT CTA GA
XbaI

LI
Y Q A
TAT CAA G

PLII
Y L D
TAT CTA C
XbaI

Fig. 1.
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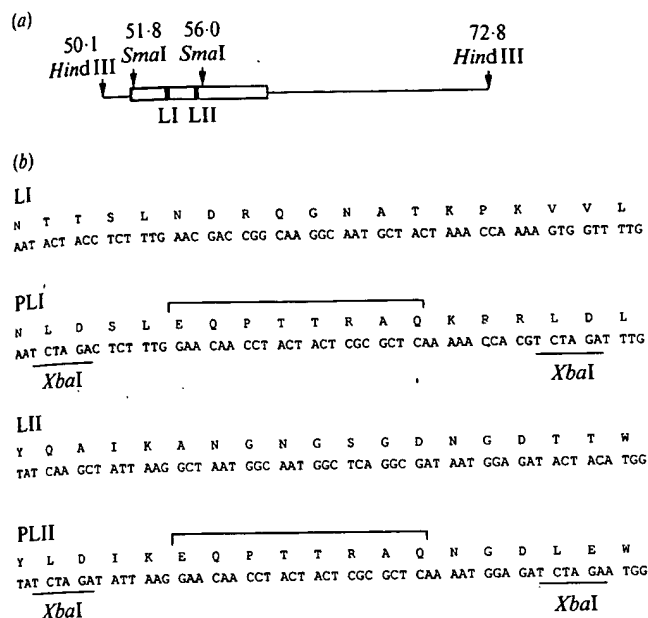


Fig. 1. Construction of adenoviruses with substitutions in the hexon. (a) The plasmid used for recombination *in vivo* contained the *Hind*III A fragment from Ad2. To modify the hexon gene (boxed) the *Sma*I fragment from 51.8 to 56.0 map units which contains sequences corresponding to both the LI and LII loops (filled boxes) was removed and cloned into M13 to provide a source of single-stranded DNA for mutagenesis. (b) The poliovirus sequence (bracketed) was inserted into the LI region to generate PLI and into the LII region to generate PLII. Following mutagenesis in M13 the *Sma*I fragment was released from the replicative form of M13 and reinserted into the *Hind*III A fragment. This reconstructed plasmid now contains a pair of *Xba*I sites that can be used for insertion of other foreign epitopes.

The adenovirus capsid contains 252 capsomers of which 240 are hexon and 12 are penton. The surface of the adenovirus virion is therefore largely composed of residues derived from hexon and as the molecule is a homotrimer any epitope expressed as part of the hexon will be repeated 720 times on the surface of the virion. Regions of hexon into which foreign sequences would be introduced were selected on the basis of knowledge of the three-dimensional structure of the Ad2 hexon (Roberts *et al.*, 1986), comparative sequence analysis of hexons from different adenoviruses (Kinloch *et al.*, 1984; Toogood & Hay, 1988; Toogood *et al.*, 1989), and the location of neutralizing epitopes on the surface of the Ad2 hexon (Toogood *et al.*, 1992). Regions in loop I (amino acids 284 to 291) and loop II (amino acids 442 to 449) were chosen as the points at which a foreign epitope could be introduced into hexon (Fig. 1). To test the feasibility of this approach part of the well characterized immunodominant antigenic site in the VP1 capsid protein (amino acids 93 to 100) of poliovirus type 3, P3/Leon/USA/37 strain (Minor *et al.*, 1983; Evans *et al.*, 1983; Ferguson *et al.*, 1985) was independently introduced into either loop I or II of the Ad2 hexon.

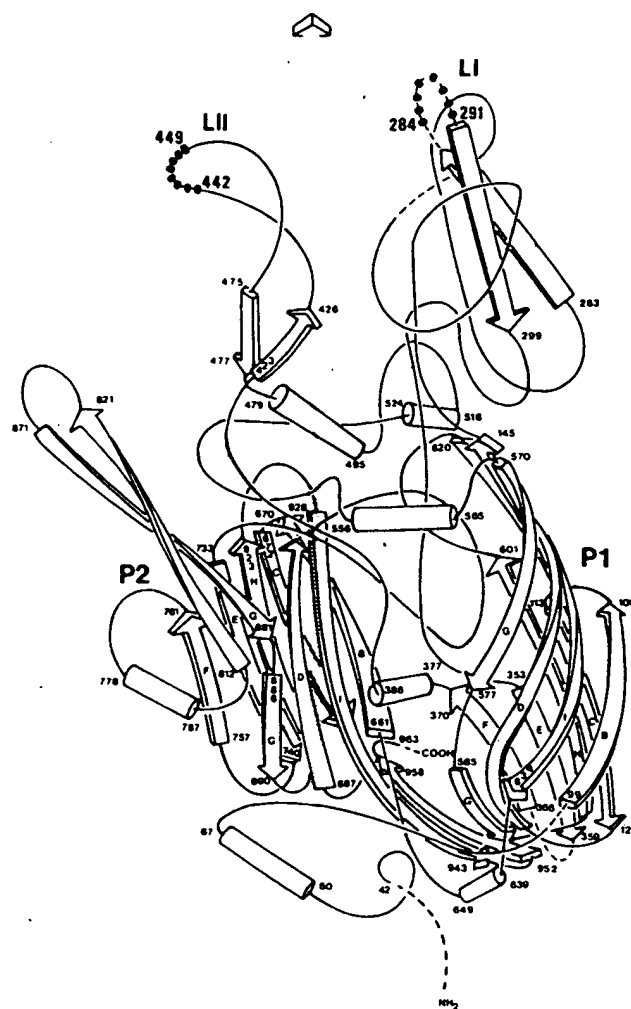


Fig. 2. Schematic diagram of the three-dimensional structure of the Ad2 hexon monomer showing location of residues altered (dots) in regions LI and LII. The two basal β -barrel domains are represented by P1 and P2 and dotted lines represent structure not resolved by X-ray crystallography (figure reproduced with permission from Roberts *et al.*, 1986).

This was accomplished by site-directed mutagenesis of an M13 subclone which was reinserted into the Ad2 *Hind*III A fragment. To aid in the selection of recombinants this plasmid was cotransfected with DNA prepared from Ad5 ts 2 which contains a temperature-sensitive lesion in the hexon gene (Boursnell & Mautner, 1981) and the virus yield was plated at the non-permissive temperature. The recombinant viruses obtained contain the poliovirus sequence Glu-Gln-Pro-Thr-Thr-Arg-Ala-Gln substituted in loop I (PLI) or loop II (PLII) and four additional amino acid changes that arise from insertion of the flanking *Xba*I sites that create a cassette into which can be inserted any sequences that are to be expressed (Fig. 1). The location of these substitutions on the surface of the hexon molecule can be determined from the three-dimensional structure of the Ad2 hexon

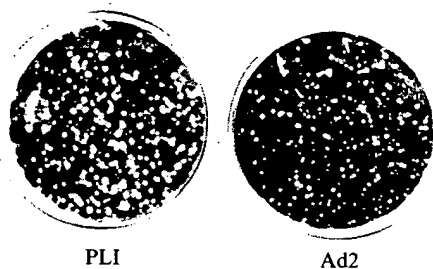
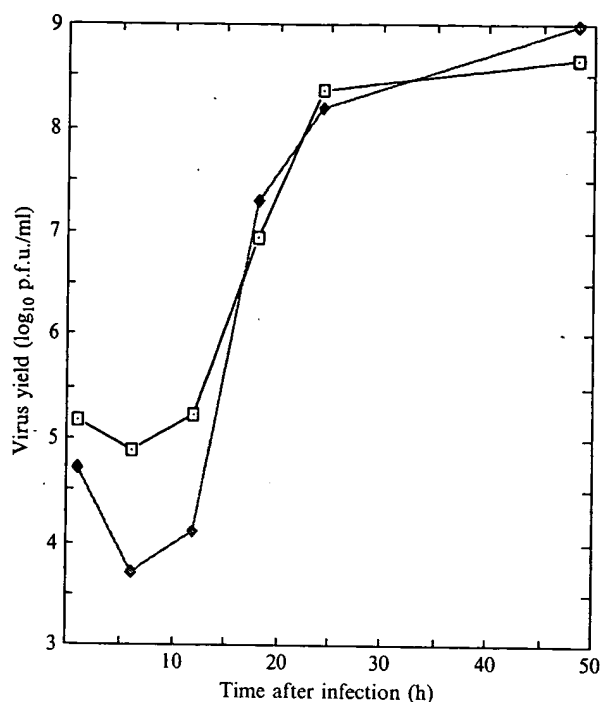


Fig. 3. Growth characteristics of Ad2 (□) and Ad5 PLI (◆). Monolayer cultures of A549 cells were infected at an m.o.i. of 5 with either Ad2 or Ad5 PLI and the virus yield was determined at the indicated times after infection by plaque assay on A549 cells (upper panel). A photograph is shown of fixed infected cells stained with crystal violet to demonstrate plaques generated by Ad5 PLI or Ad2.

(Roberts *et al.*, 1986). Fig. 2. shows the sites of substitution although given the flexibility of the loop region no attempt has been made to model the substituted amino acids into the overall structure. In both loops I and II the substituted amino acids are in a solvent-exposed position which would be predicted to be accessible to recognition by antibodies.

Growth characteristics of adenoviruses containing poliovirus epitopes

Viable viruses with substitutions in loop I (Ad5 PLI) were obtained with relative ease whereas viruses with substitutions in loop II (Ad5 PLII) were very difficult to isolate as they gave rise to very small plaques and grew very poorly. It was therefore decided to focus attention

Table 1. Recognition of Ad5 PLI by poliovirus type 3-specific antisera

Antiserum	Virus on solid phase*	
	Ad2	Ad5 PLI
Anti-hexon MAb R2/76	10000	10000
Anti-Ad5 PLI R77	5000	5000
R78	< 20	20000
R126	< 20	5000
R166	40	20000
R222	< 20	2500
R235	40	10000
		5000

* The ELISA titre is given as the reciprocal of the lowest dilution of antiserum that was at least twofold above the background reading. Poliovirus-specific antisera were raised against poliovirus type 3 VP1 amino acids 89 to 104 (EVDNEQPTTRAQKLFA; Ferguson *et al.*, 1985).

on the properties of viruses bearing substitutions in loop I. Wild-type Ad2 and Ad5 PLI were therefore compared in a one-step growth curve (Fig. 3) which demonstrates that the viruses replicate with similar kinetics and produce virus stocks of similar titre. Examination of the plaques produced by Ad5 PLI and wild-type Ad2 (Fig. 3) reinforces the conclusion that the Ad5 PLI virus has essentially wild-type growth characteristics. Loop LI was therefore identified as the preferred site for introduction of foreign sequences into the adenovirus hexon.

Recognition of Ad5 PLI by poliovirus-specific antisera

To determine whether the poliovirus sequences introduced into the hexon were presented on the surface of the virion, purified virus was used as the solid phase in an ELISA with a variety of antibodies. It should be noted at this point that although PLI is in an Ad5 background it is likely, although in this case not determined, that the hexon is derived entirely from Ad2. A MAb directed against the Ad2 hexon, MAb R2/76, gave the same titre against Ad2 and Ad5 PLI, indicating that similar amounts of Ad2 and Ad5 PLI virus had coated the plates. A similar conclusion was reached when an antiserum raised against purified Ad5 PLI (anti-Ad5 PLI) also gave the same titre for both Ad2 and Ad5 PLI. A series of antibodies raised against synthetic peptides encompassing the octapeptide sequence from poliovirus type 3 inserted into Ad5 PLI (Ferguson *et al.*, 1985) all reacted strongly with Ad5 PLI but failed to react with Ad2 (Table 1). The reactivity of the anti-peptide antibodies to Ad5 PLI indicates that the poliovirus type 3 sequences are displayed on the surface of the adenovirus virion in a form that is fully accessible to antibody.

Table 2. Neutralization of poliovirus and Ad5 PLI by poliovirus type 3-specific peptide antisera

Antiserum	Virus neutralization titre*				
	Polio 1	Polio 2	Polio 3	Ad2	Ad5 PLI
R77	< 10	< 10	160	< 10	> 20000
R78	< 10	< 10	160	< 10	> 20000
R126	< 10	< 10	320	< 10	5000
R166	< 10	< 10	20	< 10	5000
R222	< 10	< 10	10	< 10	> 20000
R235	< 10	< 10	40	< 10	> 20000

* Neutralization titres are expressed as the reciprocal of the lowest dilution of antiserum that blocks infection.

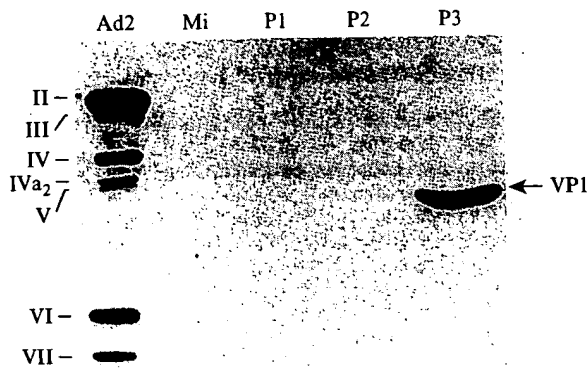


Fig. 4. Antisera raised against Ad5 PLI recognize poliovirus type 3. Western blot of cell extracts from mock-infected HeLa cells (Mi), HeLa cells infected with poliovirus type 1 (P1), type 2 (P2) or type 3 (P3) and purified Ad2 virions (Ad2). Cell extracts, Ad2 virions and prestained M_r standards (Sigma) were separated by SDS-PAGE, the proteins were transferred to nitrocellulose and reacted with a rabbit antiserum raised against heat-inactivated Ad5 PLI. Rabbit antibodies were detected with an HRP-linked second antibody and the signal was developed by enhanced chemiluminescence (Amersham). The position at which the poliovirus VP1 capsid protein migrates is indicated. Ad2 structural polypeptides detected by the antiserum are II (hexon, 103K), III (penton base, 63K although it migrates anomalously slowly in SDS-containing gels), IV (62K), IV_{a2} (51K), V (42K), VI (23K) and VII (19K).

Neutralization of Ad5 PLI by poliovirus-specific antisera

The antisera raised against the poliovirus type 3 peptide were capable of neutralizing that virus (Ferguson *et al.*, 1985). It was therefore of interest to determine whether they could neutralize the adenovirus containing the poliovirus epitope. These antisera were therefore used in neutralization tests against poliovirus types 1 to 3, Ad2 and Ad5 PLI. None of the peptide antisera have neutralizing activity against poliovirus type 1, poliovirus type 2 or Ad2 but they have, as expected, neutralizing activity against poliovirus type 3 (Table 2). In addition all of the peptide antisera efficiently neutralized Ad5 PLI (Table 2). Comparison of the neutralization titres of the

antisera for poliovirus type 3 and Ad5 PLI reveals that in almost all cases the sera are over 100-fold more active against Ad5 PLI than they are against poliovirus type 3.

Recognition of poliovirus type 3 by an antiserum raised against Ad5 PLI

To determine whether the epitope expressed on the surface of the Ad5 PLI hexon was capable of provoking an immune response, heat-inactivated purified virus was injected into rabbits and serum collected. The sera reacted vigorously in ELISA and neutralized both Ad2 and Ad5 PLI but failed to react with poliovirus type 3 in either of these tests. To determine whether antibodies had been produced against the poliovirus epitope, purified Ad2 virions and extracts from cells either mock-infected or infected with poliovirus types 1, 2 or 3 were separated by SDS-PAGE and subjected to Western blot analysis using the antisera raised against Ad5 PLI. As expected the antisera detected the structural protein of the Ad2 virion. No specific reaction was detected against uninfected cells or cells infected with poliovirus types 1 or 2 but a species corresponding to VP1 was detected in the extract of poliovirus type 3-infected cells (Fig. 4).

Discussion

We have demonstrated that viable adenoviruses can be constructed that contain a foreign sequence substituted into the exposed surface loops of the major structural protein, hexon. The regions of hexon into which these substitutions were made show type-specific variation (Toogood *et al.*, 1989) and are the sites at which neutralizing antibodies interact (Toogood *et al.*, 1992). Analysis of these regions by X-ray diffraction indicated that in addition to being located on the surface of the molecule they were disordered, displaying a considerable amount of conformational flexibility (Roberts *et al.*, 1986). Substitution into loop I produced viruses that had wild-type growth characteristics, and substitution into loop II produced viruses with impaired growth. Although these viruses were efficiently neutralized by antipeptide antisera against the poliovirus sequence, poliovirus type 3 was not neutralized by antisera raised against the adenovirus expressing the poliovirus epitope. This may be a result of the heat inactivation process which could have modified the immunogenicity of the poliovirus sequences substituted into the hexon. A number of additional explanations could also account for this observation. It is likely that the poliovirus sequence in the adenovirus hexon may be held in a quite different conformation from that in its native environment. That this is the case is suggested by the observation that the sera raised against the synthetic peptide neutralize the

recombinant adenovirus at a much greater dilution than is required to neutralize poliovirus type 3. Another possibility is that the adenovirus hexon contains an immunodominant epitope that masks any immune response to the inserted poliovirus epitope. It is also relevant that the vaccine strain of poliovirus type 3 used in these experiments is only poorly neutralized by the sera raised against the poliovirus peptide (Ferguson *et al.*, 1985 and Table 2, this report) and that the eight amino acid sequence inserted into adenovirus represents only a part of the 16 amino acid sequence that was used to generate the peptide antisera. Given the flexibility of the exposed surface loops of the adenovirus hexon it should be possible to insert considerably larger sequences of foreign material into the loop region. Comparison of the sequences of the surface loops of hexon from adenovirus types 2 and 40 reveals that relative to type 40 the type 2 hexon accommodates a 32 residue insertion of mainly acidic amino acids (Toogood *et al.*, 1989).

Although the initial construction of the plasmid containing the poliovirus sequences was rather cumbersome, further insertion of other sequences should be considerably simplified as the poliovirus sequences are flanked by *Xba*I restriction sites that are unique in the plasmid. Thus any sequences with *Xba*I compatible ends can be inserted into the cleaved plasmid thereby providing a route for the construction of adenoviruses with hexons containing defined inserts of choice. These sequences still have to be inserted into the viral genome by homologous recombination *in vivo* which is rather inefficient, but a number of strategies are now available for manipulation of the entire adenovirus genome in a plasmid background that would avoid this step (Graham, 1990). Thus the adenovirus system described here may represent a good general method for presenting small antigenic sites to the immune system. The ability of orally administered adenovirus to elicit a strong and protective immune response suggests that the chimeric viruses described here may have the ability to induce a mucosal immune response to the foreign epitopes presented on the hexon surface.

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